SCREENING TRADITIONAL CHINESE MEDICINES AGAINST ESTROGEN RECEPTORS α AND β

ZHANFANG KANG, LIHUA CHEN AND CHIWAI WONG, GUANGZHOU INSTITUTE OF BIOMEDICINE AND HEALTH, CHINESE ACADEMY OF SCIENCES, GUANGZHOU SCIENCE CITY, CHINA 510663

Here we highlight work at the Guangzhou Institute of Biomedicine and Health to screen extracts from herbs used in Traditional Chinese Medicine for effects on nuclear hormone receptor signaling.

Introduction

Nuclear hormone receptors (NHRs), like estrogen receptors alpha and beta (ERα and ERβ), are ligand-regulated transcription factors (1). A typical NHR is composed of an N-terminal ligand-independent transcriptional activation domain (AF-1), a centrally located DNA binding domain (DBD), a variable hinge region, and a C-terminally located ligand binding domain (LBD) that harbors the ligand-dependent transcriptional activation domain (AF-2; 2,3).

Traditional Chinese medicines have been used for centuries in China to treat an assortment of ailments. Traditional Chinese Medicine (TCM) involves the use of herbs and medicinal plants in combination to help alleviate specific symptoms. Some of these herb and plant extracts may modulate signal transduction pathways, such as those mediated by NHRs, although their mechanisms of action are largely unknown. Since NHRs can specifically bind to structurally distinct chemicals, and traditional Chinese medicine extracts contain chemicals that are thought to be responsible for their therapeutic actions, we sought to establish a screening assay to investigate whether certain traditional Chinese medicine extracts display agonistic or antagonistic activities on selective NHRs.

Estrogen Receptor Binding

Through their DBDs, ERα and ERβ can bind to the estrogen response element (ERE), which consists of an inverted repeat of consensus half sites AGGTCA, and activate target genes like pS2 in breast cancer cell lines and lactoferrin in endometrial cell lines in response to their cognate endogenous ligand estrogen. On the other hand, specific binding of estrogen or selective estrogen receptor modulators (SERMs), like tamoxifen and raloxifene, are mediated through their LBDs. SERMs function to either activate or suppress the activities of estrogen receptors in tissue-dependent manners. While tamoxifen functions as an antagonist to suppress estrogen-dependent growth in breast cancer cells, it functions as an agonist to mediate the skeletal protective effects in bone cells.

Phytoestrogens Influence Estrogen Response

Phytoestrogens are a diverse group of structurally distinct compounds including isoflavonoids, flavonoids, stilbenes and lignans that confer potential health benefits in cancer, cardiovascular disease, menopausal symptoms and osteoporosis (4). Phytoestrogens function at least in part as SERMs to influence the transcriptional response to estrogens in both context-dependent and tissue-selective manners (5). For example, the isoflavone genistein has been shown to bind more avidly to ERβ than ERα and suppress the expression of ER target genes like cyclin D1 and pS2 in breast cancer cells.

Preparing a Screening Assay

Using a Gal4DBD-NHR-LBD reporter system, we identified a traditional Chinese medicine extract that contained a selective ERβ agonist activity. HeLa cells at a concentration of 1.5×10^4 cells/well in 96-well plates were transfected with 0.25 µl/well lipofectamine 2000 with 25 ng/well PathDetect® pFR-Luc Trans-Reporter plasmid (Stratagene) and 5 ng/well Gal4-DBD-ERα- or ERβ-LBD expression plasmid. Various concentrations of positive control 17β-estradiol or genistein, and serial dilutions of a traditional Chinese medicine ethanol extract were added in DMEM with 5% charcoal-treated, heat-inactivated fetal bovine serum for 24 hours. Reporter activities were measured with the Steady-Glo® Luciferase Assay System (Cat.# E2510). Compared to cognate ligand 17β-estradiol, which activates both ERα and ERβ, genistein selectively activates ERβ at low concentrations and ERα at high concentrations as previously reported (1). Interestingly, we found that an ethanol extract of a traditional Chinese medicine dose-dependently activates ERβ while having no effect on ERα (Figure 1, Panels A and B). At the highest dose used, this traditional Chinese medicine extract activates ERβ as much as its cognate ligand 17β-estradiol, suggesting that the extract contains chemicals that can function as potent and specific ERβ ligands.

By scaling up the extraction, we were able to separate the chemicals into distinct fractions by HPLC. Individual fractions were then used in the reporter system, and we identified a fraction that contains ERβ-modulating activity. Genistein was also run by HPLC and found to fractionate in a different peak compared to this ERβ-modulating activity. In addition, the UV absorption spectrum of this fraction is distinct from that of genistein. By using both NMR and LC/MS methods, we confirmed the chemical identity of this fraction to be a phytoestrogen, which we temporarily named compound N (6). Highly purified compound N dose-dependently activates ERβ with an EC_{50} = 750 nM. Even at 10 µM, compound N does

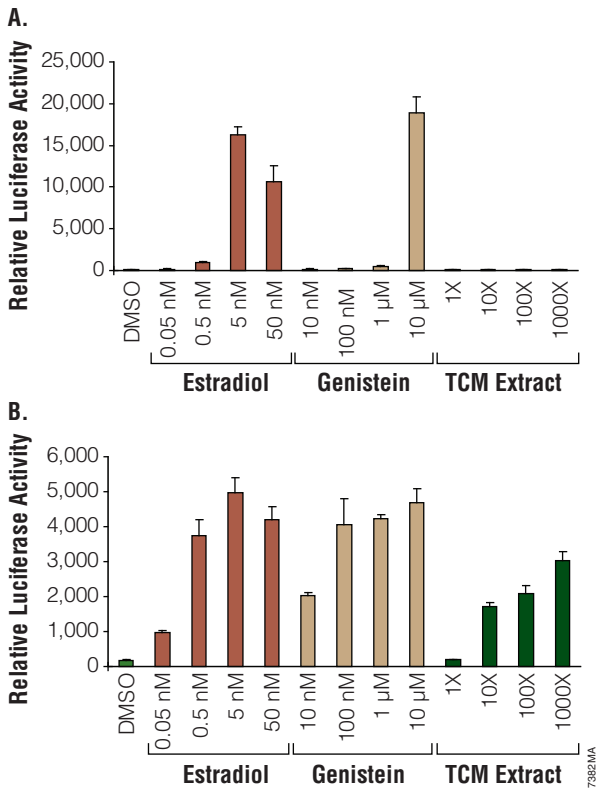

Figure 1. Comparing estradiol, genistein and a traditional Chinese medicine for their affects on ERα and ERβ. HeLa cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum seeded in 96-well plates at a density of 1.5 × 10^4 cells/well in a 37 °C incubator. Cells were transfected by 0.25 µl/well lipofectamine 2000 with 25 ng/well PathDetect® pFR-Luc Trans-Reporter plasmid (Stratagene) and 5 ng/well Gal4-DBD-ERα- (**Panel A**) or ERβ-LBD (**Panel B**) expression plasmid. Various concentrations of positive control 17β-estradiol or genistein, and serial dilutions of a TCM ethanol extract were added in DMEM with 0.5% charcoal-treated, heat-inactivated fetal bovine serum for 24 hours. DMSO was used as the carrier for estradiol. The reporter activities were measured using the Steady-Glo® Luciferase Assay System (Cat.# E2510) according to manufacturer's instructions.

not activate ERα, whereas genistein does (Figure 2, Panels A and B). In addition to testing compound N against estrogen receptors, we found that compound N does not affect the activity of peroxisome proliferator-activated receptor γ (PPARγ; data not shown).

Compound N Effects

Estrogen receptors and their ligands are believed to downmodulate NFκB activity to mediate their anti-inflammatory effects. We examined whether compound N could downmodulate the activity of NFκB on a reporter under the control of a NFκB response element. We found that compound N downmodulates the induction of NFκB activity by TNFα in HeLa cells (Figure 3, Panel A). Since iNOS is under the control of NFκB, we also examined whether compound N would downmodulate the induction of iNOS by

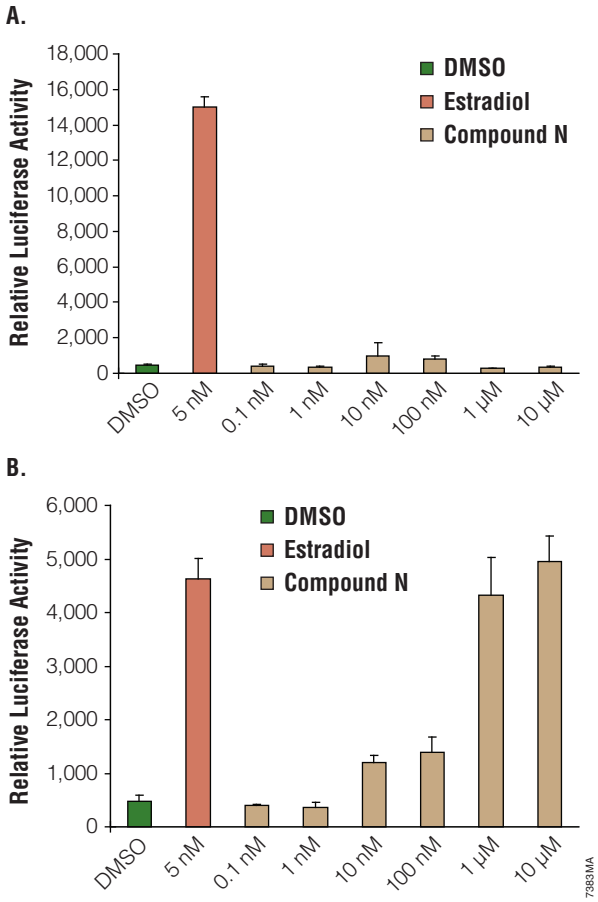

Figure 2. The effect of phytoestrogen compound N on ERα and ERβ. HeLa cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum seeded in 96-well plates at a density of 1.5 × 10^5 cells/well at 37 °C. Cells were transfected by 0.25 µl/well lipofectamine 2000 with 25 ng/well PathDetect® pFR-Luc Trans-Reporter plasmid (Stratagene), 5 ng/well Gal4-DBD-ERα- (**Panel A**) or ERβ-LBD (Panel B) expression plasmid, and 5 ng/well internal control pGL4.75[hRluc/CMV] Vector (Cat.# E6931). Positive control 5 nM 17β-estradiol or compound N, at the concentrations indicated, was then added in DMEM with 0.5% charcoal-treated heat-inactivated fetal bovine serum for 24 hours. DMSO was used as the carrier for estradiol. The reporter activities were measured using the Dual-Glo™ Luciferase Assay System (Cat.# E2920) according to the manufacturer's instructions.

monitoring nitric oxide production. We found that compound N suppressed the increase in nitric oxide production induced by LPS in the RAW264.7 macrophage cell line (Figure 3, Panel B).

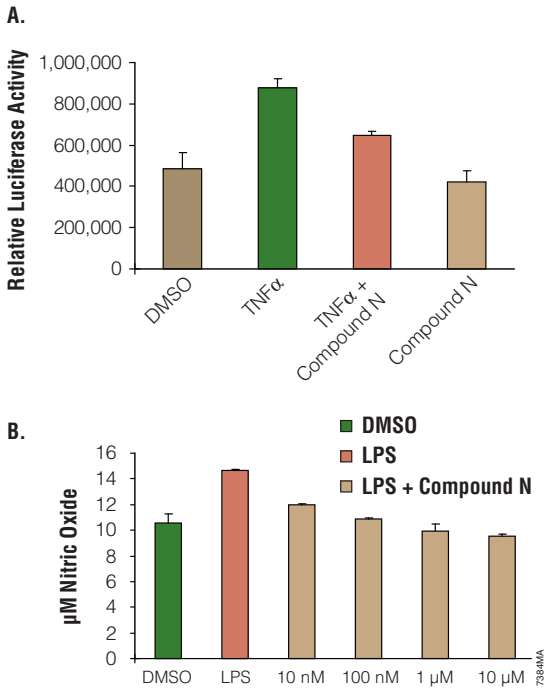

Figure 3. The effect of compound N on two cell lines. Panel A. HeLa cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum seeded in 96-well plates at a density of 1.5×10^4 cells/well at 37 °C. Cells were transfected by 0.25 µl/well Lipofectamine 2000 with 25 ng/well NFκB-luc reporter plasmid and 5 ng/well internal control pGL4.75[hRluc/CMV] Vector (Cat.# E6931) for 24 hours. TNFα was then added at 30 ng/well in DMEM with 0.5% charcoal-treated, heat-inactivated fetal bovine serum for 8 hours with or without 10 µM of compound N. The reporter activities were measured by Dual-Glo™ Luciferase Assay System (Cat.# E2920) according to the manufacturer's instructions. **Panel B.** RAW264.7 macrophages were cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum and seeded in 96-well plates at a density of 3×10^4 cells/well at 37 °C. LPS was added at a concentration of 2 µg/µl in phenol red-free DMEM with 10% charcoal-treated, heat-inactivated fetal bovine serum, and incubated for 24 hours with or without different concentrations of compound N. The amount of nitrite oxide released into the culture medium was measured by Griess Reagent (Beyotime, Jiangsu) according to the manufacturer's instructions.

Summary

By using reporter-based screening assays, we found that several herb and plant extracts used in traditional Chinese medicine contain nuclear hormone receptor-modulating activities (data not shown). Among these, we identified compound N as a selective estrogen receptor b modulator. While genistein activates both ERα and ERβ at 10 µM, compound N still retains its ERβ selectivity. The EC_{50} of compound N on ERβ is about 750 nM, which is physiologically achievable.

Some traditional Chinese medicines may exert their therapeutic effects by downmodulating inflammation. Since estrogen receptors have anti-inflammatory actions, we tested compound N from an extract of an herb used in traditional Chinese medicine to determine if it would downmodulate NFκB activity through ERβ. We found that compound N can suppress NFκB activity and inflammation-associated nitric oxide production. Collectively, these data highly suggest that traditional Chinese medicine compound N may mediate its anti-inflammatory effects through selectively binding to and activating ERβ.

References

1. Katzenellenbogen B.S. *et al.* (2000) *Breast Canc. Res.* **2**, 335–44.
2. Glass, C.K. (1994) *Endocr. Rev.* **15**, 391–407.
3. Yu, V.C. *et al.* (1992) *Curr. Opin.Biotechnol.* **3**, 597–602.
4. Ososki, A.L. *et al.* (2003) *Phytother. Res.* **17**, 845–69.
5. Cos, P. *et al.* (2003) *Planta Med.* **69**, 589–99.
6. Kuiper, G.G. *et al.* (1998) *Endocrinol.* **139**, 4252–63.

Protocols

Steady-Glo® Luciferase Assay System Technical Manual, #TM051 (www.promega.com/tbs/tm051/tm051.html)

Dual-Glo™ Luciferase Assay System Technical Manual, #TM058 (www.promega.com/tbs/tm058/tm058.html)

pGL4 Luciferase Reporter Vectors Technical Manual, #TM259 (www.promega.com/tbs/tm259/tm259.html)

Ordering Information

Product	Size	Cat.#
Steady-Glo® Luciferase Assay System	10 ml	E2510
	100 ml	E2520
	10 × 100 ml	E2550
Dual-Glo™ Luciferase Assay System	10 ml	E2920
	100 ml	E2940
	10 × 100 ml	E2980
pGL4.75[*hRluc*/CMV] Vector	20µg	E6931

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.